Short reports

Pseudomonas aeruginosa pyocyanin and 1-hydroxyphenazine inhibit fungal growth

J R Kerr, G W Taylor, A Rutman, N Høiby, P J Cole, R Wilson

Abstract

***Aim*—To examine strains of *Pseudomonas aeruginosa* for specific antifungal factors.**

***Methods*—Two clinical strains of *P aeruginosa* with strong in vitro inhibition (by cross streak assay) of *Candida albicans* and *Aspergillus fumigatus* were examined. Both strains were isolated from sputum—one from a patient with cystic fibrosis and one from a patient with bronchiectasis. Bacterial extracts were fractionated by high performance liquid chromatography and examined by ultraviolet absorbance and mass spectroscopy. Antifungal activity against *C albicans* and *A fumigatus* was determined in a well plate assay.**

***Results*—Pyocyanin was the major antifungal agent of *P aeruginosa*; 1-hydroxyphenazine also possessed activity. Pyocyanin MICs for *C albicans* and *A fumigatus* were > 64 μg/ml. These phenazines were active against nine other yeast species pathogenic for man. Preliminary experiments also suggested possible inhibition of yeast mycelial transformation in *C albicans* by pyocyanin.**

***Conclusions*—There may be a role for pyocyanin and 1-hydroxyphenazine in the prevention of pulmonary candidiasis in patients colonised by *P aeruginosa*.**

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Keywords: antifungal activity; *Pseudomonas* species; pyocyanin

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Clinical isolates of *Pseudomonas aeruginosa* from patients with and without cystic fibrosis inhibit the growth of *Candida albicans*, other *Candida* spp, and *Aspergillus fumigatus* in vitro.[1–3] In addition, despite the presence of multiple risk factors, pulmonary candidiasis is not a recognised phenomenon in cystic fibrosis patients,[1] despite the presence of *C albicans* as part of the normal flora of patients with that disease.[1] One study showed that in patients harbouring *P aeruginosa*, only 10% had positive *C albicans* skin tests, compared with 30% positivity in those free of *P aeruginosa*.[1] We addressed this issue by examining production of antifungal factors by two clinical strains of *P aeruginosa*.

Methods

HPLC FRACTIONATION AND MASS SPECTROSCOPY

We examined two strains of *P aeruginosa* cultured from sputum and showing marked fungal inhibition using the cross streak assay[2]: strain N11 (serotype O4) from a patient with bronchiectasis and strain D23 (polyagglutinable) from a patient with cystic fibrosis. *P aeruginosa* strains were cultured on brain–heart infusion (BHI) agar (Unipath) for 72 hours at 37°C in an aerobic atmosphere. After removal of macroscopic bacterial growth, the agar was cut into pieces and extracted with two volumes chloroform over three hours. Solvent was removed under vacuum and the residue subjected to reverse phase high performance liquid chromatography (HPLC) on a μBondapak C_{18} column (Waters Associates). Elution was carried out at 2 ml/min with a 20 minute linear gradient of acetonitrile:water:trifluoroacetic acid (10:90:0.04 to 70:30:0.04 vol/vol/vol). The eluate was monitored for ultraviolet (UV) absorbance at 254 nm and for antifungal activity by the well plate assay. UV spectra were obtained in either methanol or 0.1 M HCl on a Hewlett Packard 8452A diode array spectrophotometer. Mass spectrometry was carried out in the positive ion fast atom bombardment mode on a VG Quattro II mass spectrometer and by gas chromatography–mass spectrometry on a VG Trio 1000 mass spectrometer using a 15 m DB5 capillary column.

WELL PLATE ASSAY

Antifungal activity of authentic compounds (authentic pyocyanin and 1-hydroxyphenazine were synthesised by photolysis of phenazine methosulphate and phenazine, respectively[4]) and both crude and fractionated bacterial extracts was determined in a well plate assay using BHI agar flooded with a suspension of a fungal indicator strain (*C albicans* and *A fumigatus*) (10^7 cfu/ml in BHI broth) and allowed to dry. Test samples were prepared in methanol: water (1:1 vol/vol) and added to a well of 0.35 mm radius, and the plate incubated for 24 hours at 37°C. The degree of antifungal activity was expressed as the diameter of the zone of growth inhibition minus the diameter of the well.

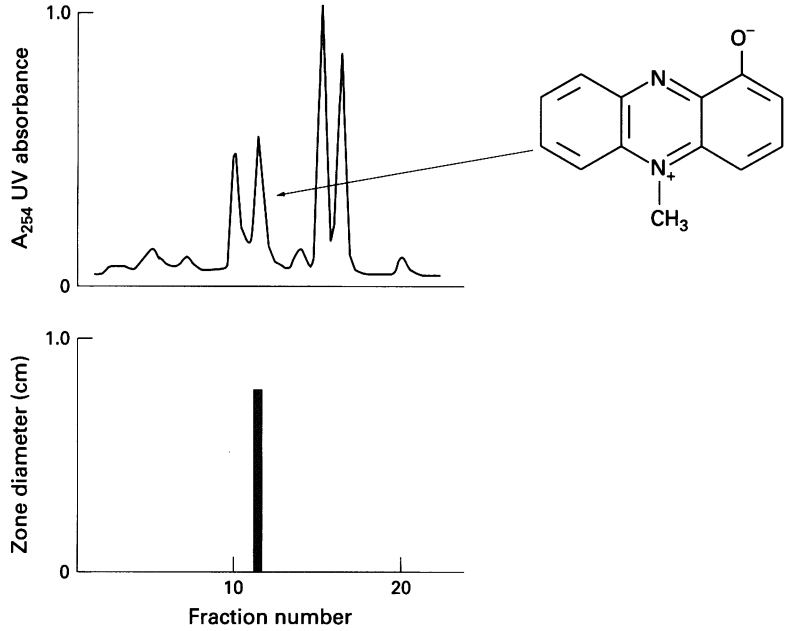

Figure 1 Reverse phase high performance liquid chromatography of chloroform extract of P aeruginosa strain N11. Antifungal activity against C albicans as determined in a well plate assay is associated with pyocyanin. Similar results were obtained with P aeruginosa strain D23.

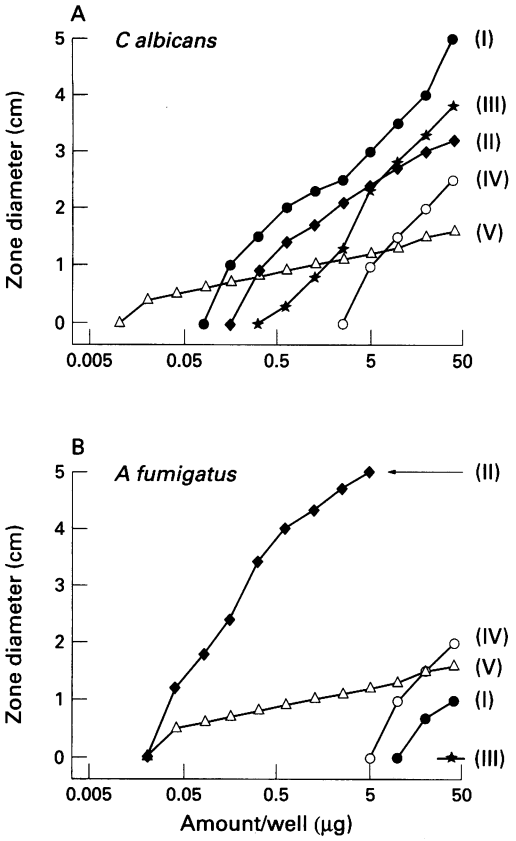

Figure 2 Dose related inhibition of (A) C albicans and (B) A fumigatus with pyocyanin (I), pyrrolnitrin (II), fluconazole (III), 1-hydroxyphenazine (IV), and amphotericin B (V). Zones of inhibition for all drugs against both fungi were complete except that of pyocyanin against C albicans.

ANTIFUNGAL SUSCEPTIBILITY STUDIES

Minimum inhibitory concentrations (MICs) were determined for authentic phenazines and antifungal drugs against *C albicans* and *A fumigatus* by broth microdilution in high resolution medium (Unipath), a method which has been shown to correlate well with NCCLS methodologies.

LIGHT AND ELECTRON MICROSCOPY OF PYOCYANIN TREATED *C ALBICANS*

Using the well plate assay on nutrient agar (Unipath) with pyocyanin (37.5 μg/well) against *C albicans* after 48 hours' incubation at 37°C, the surviving yeast cells within and outside the zone of inhibition were examined by light and electron microscopy.

Results

HPLC FRACTIONATION AND MASS SPECTROSCOPY

Following reverse phase HPLC, antifungal activity of both strains eluted at 11 minutes (fraction 11) as a single A_{254} UV absorbing compound (fig 1). The full UV spectrum of the active material showed a λ_{max} of 278 nm in 0.1 M HCl and the compound generated a positive ion fast atom bombardment mass spectrum with a protonated molecular ion cluster at m/z 211/212, allowing identification of the antifungal factor as the redox pigment, pyocyanin. Authentic pyocyanin coeluted on HPLC with the *P aeruginosa* antifungal product and was indistinguishable in terms of its UV absorbance and mass spectrum. Based on the UV absorbance of pyocyanin ($\varepsilon \sim 50\,000$), the amount of purified pyocyanin obtained from *P aeruginosa* strains N11 and D23 was 113 and 126 μg/100 ml BHI agar, respectively. The related phenazine, 1-hydroxyphenazine, eluted at 14 minutes (fraction 14), and was identified by its electron impact mass spectrum after gas chromatography (ions at m/z 196 (M+.), 168 (-CO), 142 140, 114) although this was not detected by the well plate assay.

WELL PLATE ASSAY

In the well plate assay against *C albicans*, dose dependent inhibition of yeast growth occurred at ≥ 0.15 g/well for pyocyanin (fig 2); however, even at the highest dose used (37.5 μg/well), there was still some yeast growth within the zone of inhibition. Complete growth inhibition was observed within the zone of inhibition for 1-hydroxyphenazine (≥ 4.8 μg/well), pyrrolnitrin (≥ 0.3 μg/well), fluconazole (≥ 0.6 μg/well), and amphotericin B (≥ 0.02 μg/well). The 30-fold reduction of the activity of 1-hydroxyphenazine in the well plate assay compared with pyocyanin explains why it was not detected as a *P aeruginosa* antifungal agent following HPLC. Other *P aeruginosa* products including mixed mono- and dirhamnolipids, pseudomonic acid, fluorescein, 2-heptyl-4-hydroxyquinoline and its N-oxide were inactive in the assay up to 50 μg/well.

Inhibition of *A fumigatus* growth by pyocyanin was also dose dependent but occurred at much higher levels (≥ 19 μg/well) (fig 2). In contrast to its effect on *C albicans*, pyocyanin caused complete inhibition of growth of *A fumigatus* within the zone of inhibition.

Growth of additional yeast species known to cause human infection (*C krusei, C keyfr, C guillermondii, C tropicalis, C glabrata, C lusitaniae, C parapsilosis, C pseudotropicalis*, and *S cerevisiae*) was inhibited in the well plate

assay by pyocyanin (0.6 μg/well) and 1-hydroxyphenazine (9.5 μg/well).

ANTIFUNGAL SUSCEPTIBILITY STUDIES

For *C albicans*, MICs (μg/ml) were pyocyanin 100, 1-hydroxyphenazine 25, amphotericin B 0.39, fluconazole 1.56, and pyrrolnitrin 0.39. For *A fumigatus*, the values were pyocyanin > 100, 1-hydroxyphenazine 50, amphotericin B 0.78, fluconazole > 100, and pyrrolnitrin 0.02.

There was no evidence of either synergy or antagonism between pyocyanin and 1-hydroxyphenazine against either *C albicans* or *A fumigatus*, as determined by a well plate assay on BHI agar after a 24 hour incubation in which the zones of inhibition of the two compounds intersected.

LIGHT AND ELECTRON MICROSCOPY OF PYOCYANIN TREATED *C ALBICANS*

On electron microscopy, the pyocyanin treated yeast cells showed some roughening of the surface compared with controls. On light microscopy using low power magnification (×40), there was no evidence of hyphal development in three out of three pyocyanin treated cultures of *C albicans*; hyphae were, however, numerous in control cultures.

Discussion

Several groups including our own have documented the in vitro inhibition of yeasts by *P aeruginosa*[1-3] and there are reports suggesting yeast inhibition by *P aeruginosa* in vivo in patients with[1] and without[3] cystic fibrosis. We have shown that the major antifungal activity against both *C albicans* and *A fumigatus* produced by cultures of two clinical strains of *P aeruginosa* is the redox active pigment, pyocyanin. This agrees with earlier work by Costa and colleagues who found that a crude chloroform extract of *P aeruginosa* containing pyocyanin possessed antifungal properties.[5] In addition, pyocyanin is known to have antibiotic activity against a range of bacteria, and causes alteration in neutrophil and lymphocyte function, reduction in mucociliary clearance, and inhibition of endothelial–NO interactions.[6 7]

There is a disparity between the amount of pyocyanin required for detection of growth inhibition of *C albicans* in the well plate assay (≥ 0.15 μg/well) compared with the MIC (100 μg/ml). This large difference was not observed for the other antifungal compounds against *C albicans* or for pyocyanin against *A fumigatus*. The explanation may be related to the observation that pyocyanin causes only partial inhibition of candida growth within the zone of inhibition even at high doses; all other antifungal compounds tested against *C albicans* showed complete zones of inhibition. Preliminary examination of the surviving pyocyanin treated yeast cells grown on nutrient agar by light microscopy showed a reduction in hyphal forms compared with control cultures. The yeast–mycelium transition in *C albicans* is promoted by increased levels of intracellular cyclic AMP.[8] Pyocyanin is known to reduce cAMP and ATP in human epithelial cells,[9] and a possible explanation of the increased antifungal activity of pyocyanin against *C albicans* in the well plate assay may involve a cAMP dependent reduction in hyphae formation. This could be important since the hyphal form is important in tissue adherence and invasion.

Pyocyanin is present in *P aeruginosa* infected airways secretions at concentrations up to 27.3 μg/ml,[10] which is approximately a quarter the MIC of pyocyanin for both *C albicans* and *A fumigatus*. 1-Hydroxyphenazine concentrations in these sputa may be as high as 5.2 μg/ml,[10] which is approximately one fifth the MIC of 1-hydroxyphenazine for *C albicans* and one tenth the MIÇ for *A fumigatus*. Although MIC values of pyocyanin and 1-hydroxyphenazine for both *C albicans* and *A fumigatus* are above the levels of these compounds in *P aeruginosa* infected airways secretions, they may be much higher around microcolonies of *P aeruginosa* in the lower respiratory tract where there is no dilution from non-colonised secretions and saliva. In addition, sub-MIC effects of antifungal drugs on yeasts, which are well documented, and the possibility of *C albicans* hyphal inhibition, raise the possibility that these phenazines may exert antifungal activity in the *P aeruginosa* colonised cystic fibrosis lung and explain the relative rarity of yeast infections in cystic fibrosis patients colonised by this organism.

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1 Hughes WT, Kim HK. Mycoflora in cystic fibrosis: some ecologic aspects of Pseudomonas aeruginosa and Candida albicans. *Mycopathol Mycol Appl* 1973;**50**:261–9.
2 Kerr JR. Inhibition of fungal growth by Pseudomonas aeruginosa and Pseudomonas cepacia isolated from patients with cystic fibrosis. *J Infect* 1994;**28**:305–10.
3 Kerr JR. Suppression of fungal growth exhibited by Pseudomonas aeruginosa. *J Clin Microbiol* 1994;**32**:525–7.
4 Knight M, Hartman PE, Hartman Z, *et al.* A new method of preparation of pyocyanin and demonstration of an unusual bacterial sensitivity. *Anal Biochem* 1979;**95**:19–23.
5 Costa AL, Cusumano V. Anti-mycotic activity of pyocyanine in vitro and in vivo on a pathogenic strain of Candida albicans. *G Batteriol Virol Immunol Microbiol* 1973;**66**:297–308.
6 Wilson R, Pitt T, Taylor GW, *et al.* Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro. *J Clin Invest* 1987;**79**:221–9.
7 Ras GJ, Anderson R, Taylor GW, *et al.* Proinflammatory interactions of pyocyanin and 1-hydroxyphenazine with human neutrophils in vitro. *J Infect Dis* 1990;**162**:178–185.
8 Niimi M. Dibutyryl cyclic AMP-enhanced germ tube formation in exponentially growing Candida albicans cells. *Fungal Genet Biol* 1996;**20**:79–83.
9 Kanthakumar K, Taylor G, Tsang KWT, *et al.* Mechanisms of action of Pseudomonas aeruginosa pyocyanin on human ciliary beat in vitro. *Infect Immun* 1993;**61**:2848–53.
10 Wilson R, Sykes DA, Watson D, *et al.* Measurement of Pseudomonas aeruginosa phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infect Immun* 1988;**56**:2515–17.